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Original Paper

Relationship Between the Tumour Tissue Pharmacokinetics and the Antiproliferative Effects of Anthracyclines and their Metabolites

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The intrinsic activity of anthracyclines and their metabolites was measured in order to determine whether the tumour exposure to the compounds reflects the difference in their ability to inhibit tumour growth. (Dox), 4′-epidoxorubicin (Epi-Dox), daunorubicin (Dauno), N-l-leucyl-doxorubicin (Leu-Dox) and their metabolites were analysed for their antiproliferative effects in three human malignant cell lines: MCF7, RPMI 8226 and A2780. The antitumour efficacy of equitoxic, maximum tolerated doses of the parent drugs was assessed in nude mice bearing subcutaneous (s.c.) well-established A2780 human ovarian cancer xenografts. The same doses were given to tumour-bearing mice to determine the distribution of the anthracyclines and their metabolites in A2780 tumour tissue during the first 48 h after injection. *In vitro* antiproliferative effects of the anthracyclines and their metabolites revealed a comparable activity for the parent drugs and daunorubicinol, whereas the other metabolites were at least 10-fold less active. The growth inhibition obtained in A2780 xenografts was 87% for Dox, 82% for Epi-Dox, 74% for Dauno and 97% for Leu-Dox. *In vivo*, the exposure of tumour tissue to the drug, calculated as the area under the concentration–time curve (AUC), was related to the extent of growth inhibition after correction of the AUC values for the intrinsic activity of the anthracycline. For each of the anthracyclines, the sum of the corrected AUC values (nmol/g/min) of the active compounds was calculated as 8812 for Dauno; 9320 for Epi-Dox; 10 986 for Dox and 15 163 for Leu-Dox. The sequence of increasing AUC values corresponded with the sequence of increasing growth inhibition by the four anthracyclines observed in A2780 xenografts.

Key words: anthracyclines, doxorubicin, 4′-epidoxorubicin, daunorubicin, *in vivo* efficacy, drug exposure

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INTRODUCTION

ANTHRACYCLINES, WITH doxorubicin (Dox) as an important representative, are some of the most effective anticancer agents used in the treatment of solid tumours. One major disadvantage of anthracycline therapy is the increased incidence of cardiomyopathy when certain limits of cumulative doses are exceeded, e.g. 500–550 mg/m^2 for Dox [1]. With respect to cardiotoxicity of Dox, it is currently assumed that metabolites, most probably the 13-dihydro metabolite doxorubicinol (Dol), may be more toxic than the parent drug [2–4]. Daunorubicin (Dauno), mainly used in the treatment of leukaemias, has also been found to induce cardiomyopathy [1]. Clinical evaluation of 4′-epidoxorubicin (Epi-Dox) revealed that this analogue has a slighly better therapeutic index than Dox [5]. Higher cumulative doses of this drug can be administered before cardiotoxicity limits further treatment.

Selective tumour treatment with anthracyclines may prevent the occurrence of cardiotoxicity. Since 1980, attempts have been made to synthesise leucyl-derivatives as prodrugs to be hydrolysed by an enzyme which is preferentially present in tumour tissue. N-l-leucyl-doxorubicin (Leu-Dox) is such a

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prodrug. It is synthesised by linkage of Dox to an amino acid carrier, which enables the active drug to be released after endocytosis by tumour cells or after enzymatic activation in the pericellular space [6, 7]. Indeed, in three out of four human ovarian cancer xenografts, we found that at an equitoxic dose of Leu-Dox could induce better growth inhibition than Dox itself [8].

During the past few years, we have gained experience in the estimation of the cardiotoxic potential of anthracyclines and their metabolites [4]. For each anthracycline, the normalised area under the concentration–time curve (AUC) in mouse heart tissue, and corrected for the cardiotoxicity relative to Dox as determined in the isolated mouse left atrium model [9], was used as a measure for intrinsic cardiotoxicity. With this pharmacological model, it was found that Dauno, Epi-Dox and Leu-Dox were less cardiotoxic than Dox, whereas all 13-dihydro metabolites were more cardiotoxic than the parent drugs [4]. Similarly, the AUC of the drugs and metabolites in tumour tissue, corrected for the relative potency of the compounds, may be related to the inhibition of tumour growth by anthracyclines *in vivo*. To investigate if such a relationship exists, we determined whether the antitumour activity *in vivo* may be predicted from the tumour drug exposure to the active compounds.

MATERIALS AND METHODS

Drugs

The anthracyclines were kindly provided by Farmitalia Carlo Erba (Milan, Italy), except for N-l-leucyl-doxorubicin (Leu-Dox) and N-l-leucyldoxorubicinol (Leu-Dol) which were provided by Medgenix Group (Fleurus, Belgium). All other reagents were of analytical grade.

For the HPLC analysis, separate stock solutions of each anthracycline were made in methanol (100 μM) and thereafter combined to obtain an equimolar standard mixture (10 μM) of the drugs. From this mixture, further dilutions (2.5, 1.25 μM and 250, 100, 50, 25 nM) were prepared in methanol to be used as HPLC standards. Stock solutions were diluted in tissue culture medium when drugs were investigated for the antiproliferative effects *in vitro*. For testing the antitumour efficacy *in vivo* Dox, Epi-Dox, Dauno and Leu-Dox formulated for clinical use were dissolved in water in concentrations of 2, 2, 4 and 20 mg/ml, respectively.

Cell lines and drug sensitivity

Three human malignant cell lines, derived from anthracycline-sensitive tumour types, were used to measure the antiproliferative effects of anthracyclines and their metabolites *in vitro*. MCF7 breast cancer cells [10], RPMI 8226 myeloma cells [11] and A2780 ovarian cancer cells [12] were grown in Dulbecco's modified Eagle's medium (Flow, Irvine, U.K.) supplemented with 10% heat-inactivated fetal calf serum (Gibco, Paisley, U.K.), in a humidified atmosphere containing 5% CO_2 at 37°C. Cells were screened for *Mycoplasma* contamination by using a rapid detection system with a ^{3}H-labelled DNA probe (Gen-Probe, San Diego, California, U.S.A.) and were found to be negative.

Cellular drug sensitivities were measured with a tetrazolium dye (MTT) assay [13]. On day 0, cells were seeded into 100 μl tissue culture medium in 96-well microtitre plates at 3000 cells/well, except for RPMI 8226 which was seeded at 6000 cells/well. The plates were incubated in standard conditions for 24 h. On day 1, 100 μl of freshly prepared drug dilutions in tissue culture medium were added to the wells in concentrations ranging 10 nM–10 μM or 100 nM–100 μM, depending on the sensitivity of the cells. After a 2-h incubation period, the drug-containing medium was replaced by drug-free medium using one washing step. In the case of RPMI 8226, floating cells were centrifuged first (10 min, 250*g*) before replacement of the medium. All the drug concentrations were tested in quadruplicate and experiments were repeated 2–5 times. Cells were grown for 2–3 doubling times. The antiproliferative effects were expressed as the IC_{50}, which is the concentration of the drug inducing 50% growth inhibition when compared with the growth of control cells.

Xenografts and drug sensitivity

Female nude mice (Hsd: athymic nude-nu) were purchased at the age of 6 weeks (Harlan CPB, Zeist, The Netherlands). The animals were maintained in isolation and animal handling was carried out in sterile conditions. A2780 xenografts were established by the inoculation of 1×10^7 cells s.c. in both flanks of the mice. The solid tumours arising at the inoculation site (passage 1) were transferred as tissue fragments with a diameter of 2–3 mm through a small skin incision into both flanks of 8- to 10-week-old mice. Experiments were carried out in passage 2 or later passages.

A2780 xenografts were measured twice a week in three dimensions with a slide caliper. The volume was calculated by the equation length × width × thickness × 0.5, and expressed in mm^3. At the start of treatment, tumours had a mean volume of 250 mm^3 (day 0). Groups of six tumour-bearing mice were used for control or treatment with Dox 8 mg/kg, Epi-Dox 10 mg/kg, Dauno 10 mg/kg or Leu-Dox 28 mg/kg administered i.v. weekly × 2. The doses were based on the occurrence of a mean weight loss of approximately 10% of the initial weight within one week after the first injection. Doses were established earlier as a maximum tolerated dose [8, 14], except for Dauno. Weight loss was determined by weighing the mice twice a week. For Dauno, a dose of 10 mg/kg was selected because doses of 12 mg/kg i.v. weekly × 2 appeared to induce ascites in two out of three mice.

For the evaluation of drug efficacy, the relative tumour volume was expressed by the formula V_T/V_0, where V_T is the volume on any given day and V_0 is the volume at day 0. The ratio between the mean of the relative volumes of treated tumours and that of control tumours × 100% (T/C%) was assessed on each day of measurement. Antitumour effects were expressed as the percentage of growth inhibition (100%–T/C%) and differences between groups were evaluated with the Student's *t*-test.

Distribution of anthracyclines in tumour tissue

Nude mice bearing s.c. A2780 xenografts of a mean volume of 600 mm^3 were injected i.v. with Dox 8 mg/kg, Epi-Dox 10 mg/kg, Dauno 10 mg/kg or Leu-Dox 28 mg/kg. At 1 min, 30 min, 2 h, 24 h and 48 h tumours were removed in groups of three mice per timepoint. Tissues were rinsed in NaCl 0.9%, dried and stored at –20°C. Tissues were homogenised by dismembration for 1 min at 77°K and immediately suspended in a solution containing glucose and glucaric acid-1,4-lactone to prevent decomposition of any glucuronide present [15]. Aliquots of 2 ml of 100 mg tissue (net weight) per ml solution were stored at –20°C. Analysis of anthracyclines in tissue homogenates was carried out using solid-phase extraction followed by high-performance liquid chromatography

(HPLC) with fluorescence detection, as previously published for Dox and Epi-Dox [15, 16], Dauno [17] and Leu-Dox [18, 19]. For each anthracycline and its metabolites, the AUC values were determined using the trapezoidal rule.

RESULTS

Cellular sensitivity to anthracyclines

The antiproliferative effects of the four anthracyclines and their main metabolites expressed as IC_{50} values are summarised in Table 1. The sensitivity of the cell lines MCF7, RPMI 8226 and A2780 to each of the various compounds differed only slightly. The IC_{50} of Dauno was slightly lower than that of Dox, Epi-Dox and Leu-Dox. Doxorubicinol (Dol), Epirubicinol (Eol) and Leu-Dol were less toxic than the parent drugs and mean ratios between the IC_{50} values were 31, 31, and 42, respectively. Daunorubicinol (Daunol) was approximately 14-fold less toxic than Dauno. An IC_{50} value could not be obtained for the aglycons 7d-Doxon and 7d-Dolon, as they were insoluble in the culture medium at concentrations higher than 10 μM. At 10 μM, cell growth was still at the control level. The HPLC assay was used to verify whether drug metabolism had occurred in the medium during the 2-h incubation time. The chromatograms of the media did not show metabolite peaks, except for Dauno and Leu-Dox. In these two cases, approximately 7% and 10% of the total anthracycline concentrations represented Daunol and Dox, respectively.

Antitumour activity of anthracyclines in vivo

A2780 xenografts grown s.c. in nude mice were selected to measure the antitumour effects of the four anthracyclines at equitoxic doses *in vivo*. The relative weights on day 13 after the initiation of treatment are shown in Table 2. The weight loss was not significantly different between the treatment groups. However, in three out of six mice treated with Dauno, ascites occurred which required termination of the animals on day 20.

Each drug caused an increase in tumour volume doubling time when compared with the rapid growth of control tumours (Table 2). The delay in growth was most pronounced in Leu-Dox treated tumours, but only a slight increase was observed in tumours treated with Dauno. The antitumour activity of the drugs expressed as the percentage of growth inhibition was maximal on day 20. Leu-Dox was most effective and the extent of growth inhibition was significantly different from that of Dox, Epi-Dox and Dauno ($P < 0.01$). The efficacy of Dox and Epi-Dox was similar, but significantly better when compared with the growth inhibition induced by Dauno ($P < 0.05$). Figure 1 illustrates the extent of growth inhibition obtained by the four anthracyclines in A2780 xenografts.

Table 1. Antiproliferative effects of four anthracyclines and their main metabolites

Drug*	IC_{50} in μM (n)† MCF7	RPMI 8226	A2780
Dox	2.0 ± 0.6 (3)	1.2 ± 0.3 (2)	1.5 ± 0.8 (4)
Dol	50 ± 30 (3)	58 ± 42 (2)	28 ± 8 (4)
Epi-Dox	1.4 ± 0.6 (3)	1.0 ± 0.2 (2)	0.8 ± 0.2 (4)
Eol	21 ± 12 (3)	57 ± 7 (2)	16 ± 7 (4)
Dauno	0.5 ± 0.3 (3)	0.2 ± 0.1 (3)	0.3 ± 0.2 (4)
Daunol	3.8 ± 1.1 (3)	5.3 (1)	2.4 ± 0.7 (4)
Leu-Dox	2.0 ± 0.8 (4)	1.3 ± 0.6 (2)	1.1 ± 0.5 (5)
Leu-Dol	57 ± 30 (3)	73 ± 53 (2)	45 ± 36 (4)

*Dox, doxorubicin; Dol, doxorubicinol; Epi-Dox, 4'-epidoxorubicin; Eol, epidoxorubicinol; Dauno, daunorubicin; Daunol, daunorubicinol; Leu-Dox, N-l-leucyl-doxorubicin; Leu-Dol, N-l-leucyldoxorubicinol. †Mean in μM ± S.D. (number of separate experiments).

Table 2. Antitumour effects of four anthracyclines in A2780 xenografts

Drug*	Dose mg/kg i.v.	Relative weight† % ± S.D.	Tumour volume doubling time‡	GI%§ (day 20)
Control	–	122 ± 11	2.5	–
Dox	8	88 ± 7	14.0	87%‖
Epi-Dox	10	90 ± 6	7.5	82%‖
Dauno	10	93 ± 11	3.5	74%
Leu-Dox	28	80 ± 8	23.5	97%¶

*For abbreviations see Table 1. †Weight on day 13 relative to initial weight. ‡In days from 250–500 mm^3. §Growth inhibition, maximal on day 20. ‖Significantly more effective than treatment with Dauno ($P < 0.05$). ¶Significantly more effective than treatment with Dox, Epi-Dox or Dauno ($P < 0.01$).

Distribution of anthracyclines in tumour tissue

The pharmacokinetics of the four anthracyclines in A2780 xenografts revealed that the peak Dox concentration in tumour tissue of 4.68 nmol/g decreased to 2.84 nmol/g and that of Epi-Dox of 4.16 nmol/g decreased to 2.33 nmol/g 48 h after injection. After administration of Dauno, tissue concentrations increased from 4.11 nmol/g to 5.46 nmol/g during the first 2 h with a rapid decrease thereafter. Daunol was formed and the highest level of 1.75 nmol/g was measured at 24 h. Kinetics of Leu-Dox showed an initial high concentration of 24.30 nmol/g at 1 min, followed by a rapid decrease to undetectable levels at 24 h. Dox formation could be measured at 1 min (0.56 nmol/g) and its concentration was highest 24 h (5.54 nmol/g) after administration of Leu-Dox. The AUC values of the anthracyclines and their metabolites were calculated over the first 48 h (Table 3).

Kinetics in tumour tissue versus growth inhibition

The antiproliferative effects of the compounds in A2780 cells *in vitro* indicated comparable activity for the parent drugs and Daunol, whereas the other metabolites were at least 10-fold less active. This means that for each anthracycline, the sum of the AUC values (nmol/g/min) of the active compounds represents the exposure of the tumour to these compounds, 10 986 for Dox, 9320 for Epi-Dox, 8812 for Dauno and 15 163 for Leu-Dox. The sequence of decreasing AUC values of the active compounds Leu-Dox > Dox > Epi-Dox > Dauno corresponds with the sequence of growth inhibition obtained in A2780 xenografts.

DISCUSSION

Our objective was to compare the pharmacokinetics of four anthracyclines and their metabolites, given at equitoxic doses to nude mice bearing human tumour xenografts, in order to assess whether the AUC of the compounds reflects the difference in their inhibition of tumour growth. As an example, we used A2780 human ovarian cancer xenografts grown s.c. in the nude mouse. Because the AUC denotes the exposure of

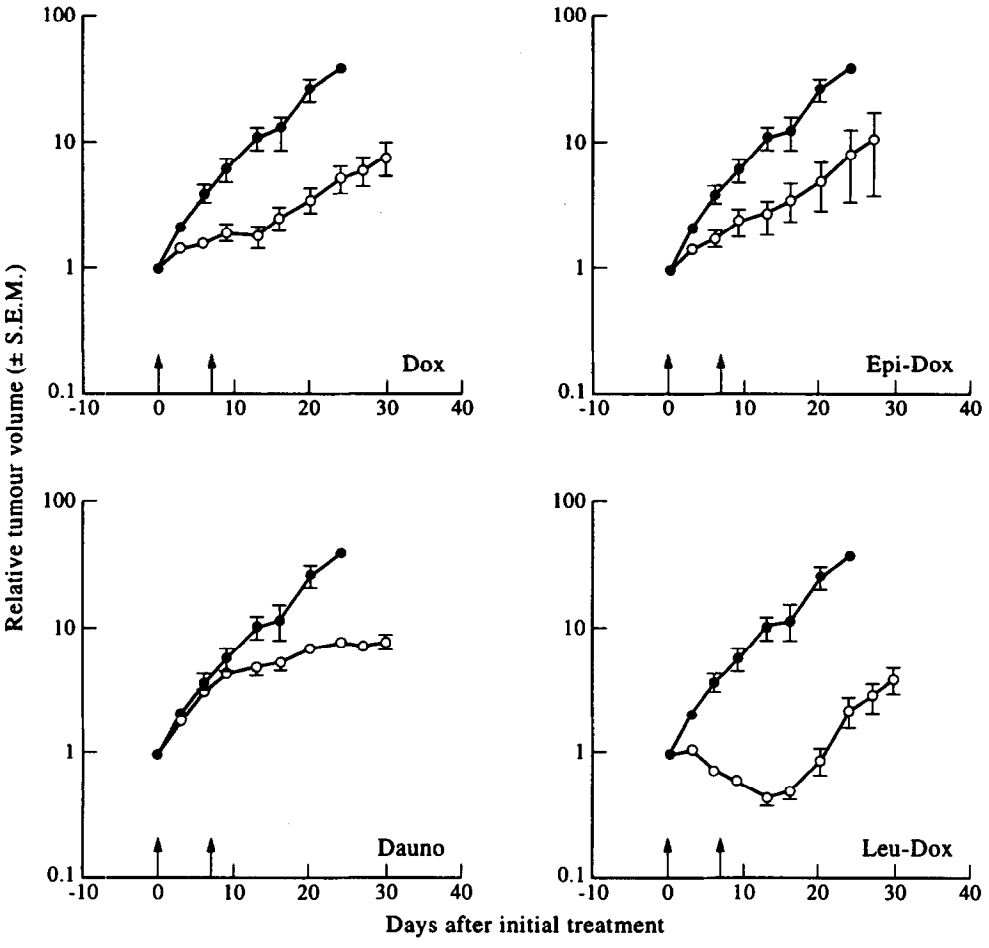

Figure 1. Growth curves of A2780 s.c. human ovarian cancer xenografts as control (●) or treated i.v. weekly × 2 (○) with doxorubicin (Dox) 8 mg/kg, 4'-epidoxorubicin (Epi-Dox) 10 mg/kg, daunorubicin (Dauno) 10 mg/kg or N-l-leucyl-doxorubicin (Leu-Dox) 28 mg/kg.

Table 3. AUC values of anthracyclines and their metabolites† in A2780 xenografts*

Compound	Dox 8 mg/kg i.v.	Compound	Epi-Dox 10 mg/kg i.v.	Compound	Dauno 10 mg/kg i.v.	Compound	Leu-Dox 28 mg/kg i.v.
Dox	10986	Epi-Dox	9320	Dauno	4762	Leu-Dox	3164
Dol	86	Eol	0	Daunol	4050	Leu-Dol	0
Dolon	31	7d-Don	0.3	Daunolon	0.3	Dox	11999
						Dol	0
						Dolon	20
						7d-Dolon	482
						Don	13

*AUC values calculated over the first 48 h (nmol/g/min). †For abbreviations see Table 1.

the tumour to a drug, this pharmacokinetic parameter was chosen to predict the antitumour activity of the drug *in vivo*. The AUC values of the drugs can only be used for this purpose, when the relative potency of each drug is taken into account. Therefore, we determined the IC50 values for each individual anthracycline and its metabolites in three different human malignant cell lines. The MTT chemosensitivity assay was chosen as the *in vitro* test system, because of its simplicity and because it requires minimal amounts of drugs. Incubations of 2 h were preferred over long-term exposure to prevent degradation and/or metabolism of the compounds in the culture medium and the cells. Nevertheless, drug metabolism was observed in two cases, i.e. Daunol from Dauno and Dox from Leu-Dox. As Daunol and Dox represented low amounts of the total anthracycline concentration in the tissue culture medium, it may be anticipated that the IC_{50} values of the parent drugs were only slightly overestimated.

The antiproliferative effects of the anthracyclines and their metabolites showed a consistent pattern in the three human malignant cell lines tested. All 13-dihydro metabolites were less potent than the parent compounds and mean ratios between the IC_{50} values were 31 for Dox>Dol, 31 for Epi-

Dox/Eol, 14 for Dauno/Daunol and 42 for Leu-Dox/Leu-Dol. Schott and Robert [20] have also used 2-h incubations for 13-dihydro metabolites and parent compounds in rat glioblastoma cells and obtained comparable results with ratios of 33, 9 and 11, respectively, for the first three sets of compounds mentioned. Ferrazzi and colleagues [21], comparing Dox, idarubicin, Dol and idarubicinol with respect to the induction of DNA damage, determined IC_{50} values of 0.2 and 4 μM for Dox and Dol, respectively.

Although *in vitro* chemosensitivity assays do not always allow for a prediction of the *in vivo* antitumour efficacy of a drug, their use in the determination of the relative potency of analogues and their metabolites seems to be justified [22]. Upon comparison of the activity of the anthracyclines *in vitro*, we would expect Dauno to be the most effective in treating cancer *in vivo*. Our *in vivo* data on the superiority of Leu-Dox > Dox > Epi-Dox > Dauno appears to relate better to the clinic than the data obtained *in vitro*, because Dox and Epi-Dox have a wide spectrum of activity in solid tumours whereas treatment with Dauno is mainly limited to leukaemias [1]. Moreover, it has been shown that the maximum tolerated dose of Leu-Dox in patients is 3-fold higher than the standard dose of Dox [23]. Unfortunately, it is not known whether Leu-Dox shows better responses in cancer patients, as comparative trials have not been performed.

The growth inhibition observed in A2780 human ovarian cancer xenografts when anthracyclines were given at equitoxic, maximum tolerated doses showed the following sequence in efficacy: Leu-Dox > Dox > Epi-Dox > Dauno. This sequence might also have been expected when xenografts of solid tumours with different sensitivities to anthracyclines had been added to our *in vivo* experiments. With the use of the same doses and the same schedule, we have previously reported that the growth inhibition induced by Epi-Dox is slightly, but significantly lower than that of Dox in four out of eight human ovarian cancer xenografts [14]. We have also reported that the efficacy of Leu-Dox is superior to that of Dox in three out of four xenografts [8]. Currently, analogue screening in human tumour xenografts can be considered to be highly valuable for decisions on whether to proceed to the clinic, especially for platinum compounds and anthracyclines [24]. The better relationship between the *in vivo* data and clinical observations have to be attributed to the role of drug metabolism and host toxicity which can be taken into account. One should keep in mind that a lesser dose-limiting toxicity of a relatively less potent analogue in the clinic may permit the administration of higher doses and eventually lead to a better antitumour effect.

In most *in vitro* experiments, comparative uptake studies in malignant cells are based on the exposure to similar concentrations of drug for a defined period of time [20, 25]. Indeed, the extent of cellular uptake of anthracyclines has been related to the degree of DNA synthesis inhibition, because Dauno has better therapeutic characteristics than Epi-Dox or Dox as measured in rat glioblastoma cells [20]. Dox appears to be 3- to 4-fold and 7- to 10-fold more active than Leu-Dox in MCF7 breast cancer cells and in K562 leukaemia cells, respectively, which could be explained by a difference in incorporation of the drugs into the cells [25]. However, intracellular drug concentrations do not always parallel differences in the intrinsic activity between anthracyclines. From our point of view, additional measurement of tumour tissue levels of the active compounds obtained from *in vivo* drug exposure may better predict differences in activity between anthracyclines. Because of the high intracellular uptake of anthracyclines, tissue concentrations may be considered to represent intracellular concentrations.

At the maximum tolerated dose, the AUC values of anthracyclines and their metabolites in tumour tissue might be used to estimate the activity of the four drugs in the treatment of solid tumours. For that purpose, the AUC values have to be corrected for the intrinsic activity of the various compounds *in vitro*. When the AUC values are added up for the active compounds, the total AUC values decline in the order of Leu-Dox > Dox > Epi-Dox > Dauno. This order corresponds with the sequence of antitumour activity determined for the four drugs in A2780 xenografts, which means that the *in vivo* activity of anthracyclines is related to the tumour exposure of the parent drug and its metabolites provided that the intrinsic activities are taken into account. These findings may possibly be of help in the further development of anthracycline analogues and give insight into the relative contribution of the parent drug and its metabolites to the *in vivo* antitumour effects.

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